The application of an *Agrobacterium*-mediated *in planta* transformation system in a *Catharanthus roseus* medicinal plant

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Abstract: The lack of an efficient protocol for the transformation of *Catharanthus roseus*, as an important medicinal plant, causes obstacles in the genetic engineering programmes of this plant. Therefore, the present study was conducted to examine a convenient and reliable system for generating stable transgenic lines based on the *in planta* method. To do so, two different *in planta Agrobacterium* infection systems were investigated. The transgenic lines were produced through ovary injection and shoot apical meristem (SAM) immersing methods. The presence and expression of the *GFP* transgene in the T0 and T1 lines were confirmed by PCR and RT-PCR. Moreover, the ELISA test confirmed the *GFP* expression in the transgenic lines. Apart from the superiority of the ovary injection over the SAM inoculation due to the normal growth and healthy appearance, it also showed a significant difference from the point of the confirmed transgenic plant numbers. *GFP* integration was confirmed in 6 out of 50 T1 plants from the ovary injection and only 3 out of 150 plants generated from the SAM inoculation. In addition to the comparison of these two separate methods, we reached a new convenient *in planta* system by a 12% transformation rate for generating the *C. roseus* transgenic lines. According to the result of this study, ovary injection can be introduced as a novel, facile, and stable method for the *Catharanthus* transformation programme.

Keywords: gene expression; *GFP* gene; ovary injection; shoot apical meristem infection; transformation rate

Catharanthus roseus (L.) G. Don is an important dicotyledon medicinal plant that belongs to the family Apocynaceae (Magnotta *et al.* 2006). *C. roseus* is known for its pharmaceutical important terpenoid indole alkaloids (TIAs) such as vinblastine, vincristine and ajmalicine which have anticancer, antidiabetic and hypertension control properties (Guimaraes *et al.* 2012). The yield of these natural compounds is meagre in the wild-type *C. roseus* plant. Therefore, the genetic manipulation for increasing the yield of the main alkaloids is a crucial breeding goal in this plant.

Since regeneration of *Catharanthus* as a recalcitrant plant face many limitations, most of the alkaloid enhancement projects in *C. roseus* have been carried out in cell suspension and hairy root culture systems (Canel *et al.* 1998). Nevertheless, the tissue compartment dependent production of the terpenoid indole alkaloids in *C. roseus* inhibits reaching a desirable yield of these compounds in the cell suspension or hairy root culture systems (Zhao *et al.* 2013). The lack of optimised transformation and regeneration methods for *C. roseus*, has caused many obstacles

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in the genetic manipulation of this plant. Recently, some investigations have been undertaken to produce transgenic *Catharanthus* lines via the standardisation of different factors in the tissue culture system. Wang *et al.* (2012) and Alam *et al.* (2017) presented an optimised protocol for the production of transgenic *Catharanthus* lines. Despite their success, like most of the other reports on this issue, the protocol was based on the indirect regeneration from callus cultures which increase the risk of the genetic variation (Muzamli *et al.* 2016). Verma and Mathur (2011) studied the effect of different factors such as the co-cultivation duration and sonication time on the direct regeneration of the transformed *Catharanthus* lines via bud organogenesis from the leaf explants. However, the necessity of developing a convenient, repeatable and cost-effective transformation system still exists for the transformation of such an important medicinal plant.

The *in planta* transformation method is a tissue culture independent strategy for the transgenic line production in which the plantlets are infected by *Agrobacterium* and are generated free of laborious and time-consuming regeneration processes. Apart from the simplicity and time savings, the somaclonal diversity is reduced in this method (Jaganath *et al.* 2014). This method has been successfully used in many plants such as wheat (Supartana *et al.* 2006), cotton (Keshamma *et al.* 2008), tomato (Shah *et al.* 2015), *Vitis* spp. (Ben-Amar *et al.* 2013), jatropha (Jaganath *et al.* 2014), and *Sorghum bicolor* (Yellisetty *et al.* 2015). However, *in planta* transformation has rarely been used in medicinal plants and has not been applied in *C. roseus* until now. To overcome the common transformation limitations of *C. roseus* through the tissue culture-based regeneration and take advantage of the *in planta* transformation technique, we established an *in planta* transformation method using the shoot apical meristem (SAM) and ovary tissues and compared these two methods for the first time.

MATERIAL AND METHODS

The plant material. *C. roseus* seeds of a rose-pink cultivar were obtained from the Plant Virology Research Center of Shiraz University. For sterilisation, the seeds were surface sterilised with 70% ethanol for 90 s followed by a soaking in sodium hypochlorite (30%, 15 min), rinsed five times with sterile distilled water and then germinated on a half strength solid MS (Murashige and Skoog) medium at 24°C in dark

conditions. We used prepared Sigma MS basal medium powder with 3% added sugar and 0.6% agar at a pH of 5.8 which was autoclaved for all the necessary parts in this study. The shoot apical meristems from the intact three-day-old seedlings were used for the infection with *Agrobacterium* (Shah *et al.* 2015). For the ovary injection, the seeds were grown in pots and kept up to the flowering stage in a greenhouse under optimised light and moisture conditions.

The expression vector and bacterial strain. A binary pBI121 vector, harbouring the *GFP* gene under the control of the CAMV35S promoter and NOS terminator, was used for the transformation. The vector also contains the *nptII* (kanamycin resistance) gene under the control of the NOS promoter and terminator. The vector was kindly provided by the Plant Protection Department of Ferdowsi University of Mashhad. The *Agrobacterium tumefaciens* C58 strain was transformed with the PBI121/*GFP* construct through the electroporation method and used for the subsequent transformation.

The *in planta* transformation method. A single colony of *A. tumefaciens* was grown in 20 ml of liquid LB with 50 mg/l kanamycin and 50 mg/l rifampicin at 28°C for 24 h. The cell suspension was centrifuged at 4500 rpm for 10 min. The pellet was resolved in the MS liquid medium and used for the plant infection until it reached $OD_{600}0.5$. The bacterial suspension culture was also supplemented with 100 µM acetosyringone 1 h before the inoculation.

By using a narrow syringe (1cc) needle, the shoot apical meristems were pierced lightly and then immersed in the *Agrobacterium* suspension for 40 min by shaking at 50 rpm in a horizontal shaker. The *Agrobacterium* suspension infection was directly undertaken by immersing the three-day old germinated seeds containing the intact apical meristem and the surrounding region. Afterward, they were transferred to the MS solid medium in dark conditions for two days for the co-culture with the *Agrobacterium*. Then, the infected shoot apical meristems were washed with 400 mg/l cefotaxime and transferred to pots that contained the autoclaved sterile soil (clay 1 : cocopeat 0.5 : perlite 0.5). The T0 plant seedlings were irrigated regularly and kept in a growth chamber under controlled conditions (16 h light/8 h darkness, 24°C). The two-week-old seedlings were moved to pots with a normal soil in the greenhouse at 25–29°C for further growth and analysis.

For the ovary transformation part, we used four-month old plants which were grown in a greenhouse.

The bacterial suspension was injected into the ovary of the flowers, one day after the flower opened, using a sterilised syringe needle (1cc). To use the precise location for the ovary injection, we made a cross section on the flower and observed the exact location where the ovary contained ovules at the connection base of the sepals. The injection was performed on this intact part of the newly opened flowers. Then the infected flowers on the plant were marked. The 300 obtained T1 seeds from the harvested pods were grown on the selection medium (MS + 200 mg/l kanamycin) for three weeks until the seeds germinated, and the seedlings grew. Then, the surviving green seedlings were transferred to a sterile autoclaved soil (clay 1 : cocopeat 0.5 : perlite 0.5), and moved to a normal soil two weeks later, similar to the SAMs. The culture process on 200 mg/l kanamycin medium was just used to avoid growing enormous plants in the greenhouse and to facilitate the handling of a large number of plants in the ovary injection method for further screening. The procedure of the *C. roseus* transformation is demonstrated in Figure 1 for both methods.

The DNA isolation and the PCR analysis. DNA was isolated from the leaves of the putative transformed plants which showed kanamycin resistance and untransformed plants using the CTAB method (Gawel & Jarret 1991). The PCR was performed with *GFP* gene-specific primers (F-*GFP*, 5'-GTT GAA TTA GAT GGT GAT G -3' and R-*GFP*, 5'-GAT ATG GTT GTC TGG TAA- 3'). The length of the amplified fragment was 555 bp. The PCR was carried out at 94°C for 5 min, followed by 35 cycles of amplification (94°C for 1 min, 59°C for 1 min, and 72°C for 50 s) with a final extension of 72°C for 10 min.

The RNA extraction and the RT-PCR analysis. The total RNA was extracted from the leaves of the transgenic and control plants using a Denazist kit (Denazist, Iran). The cDNA was synthesised from 3 μg of total RNA using a cDNA synthesis kit (AmpliSens, Russia) after treatment with a DNase enzyme (Thermo Scientific, USA). The RT-PCR analysis was conducted using *GFP* gene-specific primers with the same programme except for the annealing temperature, which was set at 52°C.

The ELISA assay. An indirect ELISA assay with a specific antibody named anti-*GFP* (Biolegend, USA) was conducted to ensure the *GFP* gene is expressed and active protein is produced. The total protein was extracted from the leaf tissues by a 50 mM K_2HPO_4 buffer (pH = 7), followed by centrifuging for 30 min at 13 000 rpm at 4°C. The protein concentration was determined by the Bradford method (Kruger 2002). The ELISA test was carried out with a 30-μg protein extract in three replicates for each sample. The non-transgenic protein extract was used as a negative control and the protein extraction buffer was used as a blank. Finally, the colour intensity was read at 450 nm by the ELISA reader. To determine the transgenic and non-transgenic plants according to the ELISA test, we used the intensity quantity of the control plant ± three times the standard deviations value (Wang *et al.* 2007). Any plant with an optical absorption higher than this value was considered as a positive transgenic plant expressing the *GFP* protein.

The statistical analysis. In order to statistically study the number of remaining *Catharanthus* plants in two different *in planta* systems, we performed a Fisher test with the GraphPad software. Particularly, the Fisher exact test was used to compare the

Catharanthus roseus in panta transformation
Ovary injection method

Agrobacterium injection into the ovary
of newly opened flowers

One month

Harvest seeds from pods

Culture seeds on the selection medium
(MS + 200 mg/l kanamycin)

Three weeks

Green seedlings transferred to autoclaved
sterile soil

Catharanthus roseus in panta transformation
SAM inoculation method

Surface sterilisation of seeds

Seed culture on half strength solid
MS medium

Three days

Shoot apical meristem inoculation
with *Agrobacterium* suspension

40 min

Transfer to co-culture MS medium

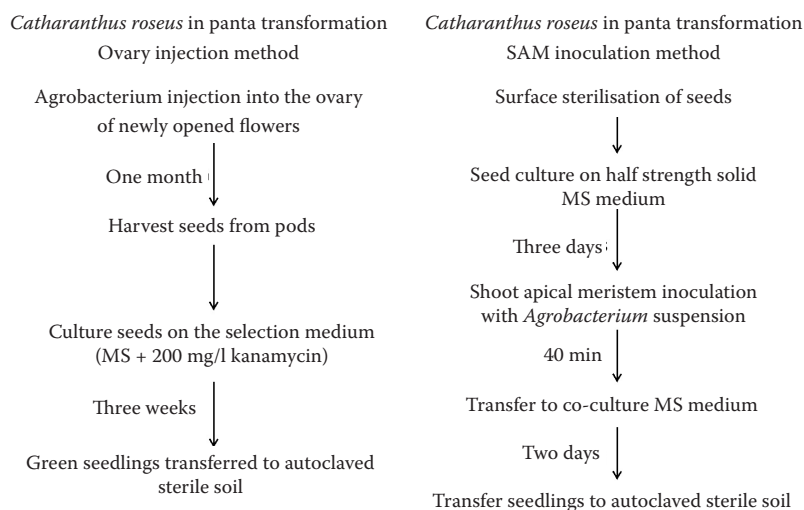Two days

Transfer seedlings to autoclaved sterile soil

Figure 1. The procedure of the *Catharanthus roseus* transformation

Table 1. Fisher's exact test for comparing the shoot apical meristem and the ovary infection methods of the *in planta* transformation in *Catharanthus roseus* using the number of generated and confirmed transgenic plants.

Tissue	No. of infected organs	No. of plants (%)	
		survived	transgenic
Shoot apical meristem	360	150 (41.66)**	3 (2)
Ovary	300	50 (16.66)	6 (12)*
Total	660	200	9

*, **$P < 0.05, 0.001$

number of generated seedlings and the number of confirmed transgenic lines between the two different SAM inoculation and ovary injection transformation methods.

RESULTS

The *in planta* transformation of the shoot apical meristem. To implement the SAM inoculation method, 360 SAMs were inoculated with *A. tumefaciens* harbouring the *GFP* construct while just 150 T0 developed seedlings remained for the further analysis after the infection and these plants were transferred directly to the sterile soil. Since the shoot apical meristems were delicate and extremely fragile at their basal part on the seeds, some of them died and some others did not grow in the autoclaved soil. As a result, 41.66% of all the infected seedlings survived in the soil (Table 1). Although these plants remained alive, they were intensively weak, tender and also exhibited very slow growth.

The *in planta* transformation of the ovary. In the second method, *A. tumefaciens* harbouring the GFP construct was injected into the ovaries of about 300 newly opened flowers by a sterilised syringe needle (Figure 2a). The obtained 300 seeds from the injected ovaries after the pod formation were cultured on the kanamycin (200 mg/l) selection medium to primarily screen the putative transgenics. After this screening, 50 T1 green seedlings were achieved and the plants were transferred to the autoclaved sterile soil (Figure 2b, c). From the total seeds obtained from the injected ovaries, 16.66% of the plants were germinated and grown in the selection medium (Table 1). According to the statistical analysis by the Fisher test, the number of generated lines in the ovary injection compared with the SAM inoculation was significantly different ($P < 0.001$).

The PCR analysis. The results of the PCR with the *GFP* specific primers approved the *GFP* amplification in some putative transgenic lines (Figure 3a, b). According to the results, 2% (3 out of 150) of the T0 and 12% (6 out of 50) of the T1 surviving plants from the SAM inoculation and ovary injection, respectively, were confirmed by the PCR. The statistical analysis of the confirmed transgenic lines statistically demonstrated the significant difference between these two pathways ($P < 0.05$) (Table 1).

The RT-PCR analysis and the ELISA test. The RT-PCR results confirmed the expression of the *GFP* gene in the *Catharanthus* leaves (Figure 4). Besides, the *GFP* protein production was confirmed by the
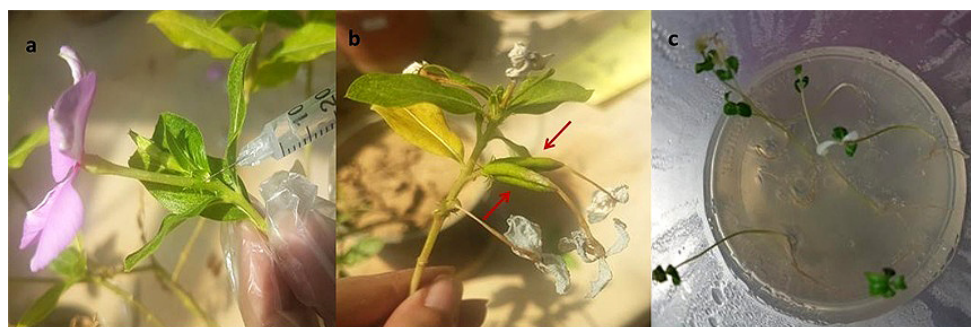

Figure 2. The *in planta* transformation of the *Catharanthus roseus* ovaries: the bacterial injection into the ovaries with a syringe and needle (a), pod formation after the infection (b) and the T1 seedlings from the seeds on the selection medium (c); the selected seedlings were then transferred to the soil
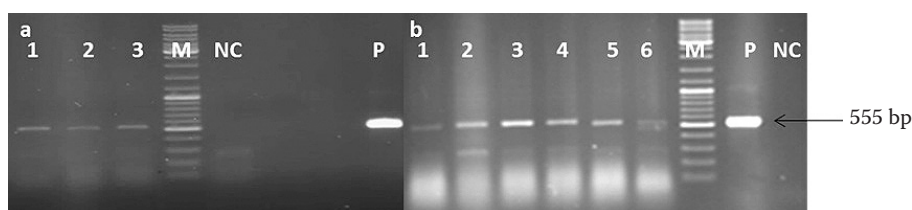
Figure 3. The PCR analysis of the *Catharanthus roseus* plants obtained by the *in planta* transformation of the shoot apical meristem and ovaries: (a) the plants originating from the shoot apical meristem infection; 1–3 the transgenic lines; M – the 100 bp DNA ladder; NC – the non-transgenic plant; P – the positive control; vector PBI121 + GFP; (b) the plants originating from the ovary injection; 1–6 the transgenic lines; M – the100 bp DNA ladder; P – the positive control; NC – the non-transgenic line
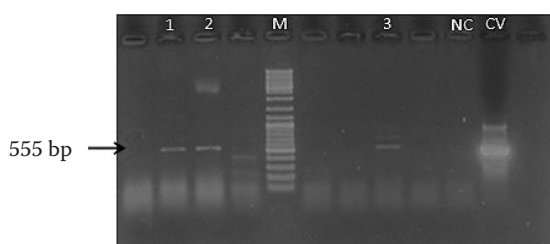

Figure 4. The RT-PCR analysis of the *GFP* gene expression in the *Catharanthus roseus* plants originating from the *in planta* method; the RT-PCR was done for the *GFP* gene with the specific primers amplified 555bp fragment; 1–3 – the transgenic lines; M – the 100 bp DNA ladder; NC – the non-transgenic plant; CV – the positive control vector PBI121+*GFP*
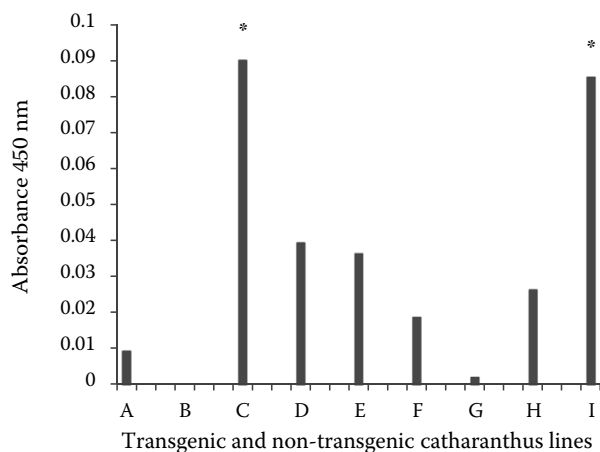

Figure 5. The ELISA assay of the molecularly confirmed transgenic *Catharanthus* plants which expressed the *GFP* gene and the control plant: C and I – the confirmed transgenic *Catharanthus* lines by ELISA, A – the negative control or the wild-type plant, B – the blank and D–H non-transgenic plants according to the ELISA analysis; the alphabets on the *x* axis show the individual lines; the absorbance intensity shown on the *y* axis is the average absorbance of the three replicates for each *C. roseus* line

specific anti-*GFP* using the ELISA assay, as well. For both the SAM and ovary methods, the *GFP* expression at the RNA and protein level was examined in all the transformed lines. Figures 4 and 5 demonstrate the results of these tests in some transgenic lines generated via both methods. From the total nine PCR positive transgenic lines, 3 and 2 were confirmed by the RT-PCR and ELISA, respectively.

DISCUSSION

Different studies have proved that the *C. roseus* transformation through common genetic engineering systems, which are mostly dependent on the tissue culture, face several barriers, particularly in the plant regeneration phase (Dhandapani *et al*. 2007; Makhzoum *et al*. 2015). Although researchers have undertaken comprehensive investigations on the establishment of a standard protocol for the regeneration of *C. roseus* like reports by Wang *et al*. (2012) and Alam *et al*. (2017), the lack of appropriate explant types for the direct regeneration and the low percentage of the transformation and abnormal phenotypes necessitates the establishment of a standard method for the *Catharanthus* transformation (Choi *et al*. 2004). In order to overcome the limitations of the gene transfer into *Catharanthus* through a tissue culture regeneration system, we investigated the possibility of using an *in vivo Agrobacterium*-mediated plant transformation or an *in planta* method instead of a conventional *in vitro* culture regeneration for the first time. According to the different reports, the efficiency of the gene transfer by use of the *in planta* method is increased in comparison with the typical methods, which are dependent on the plant tissue culture system. The *in planta* method was examined firstly in *Arabidopsis*. The vacuum infiltration method and floral dip are the two ways the *in planta* transformation were tested in *Arabidopsis*

with less than 1% transformation efficiency and then developed for many other species (Feldmann & Marks 1987; Clough & Bent 1998). Supartana *et al.* (2006) applied the *in planta* method for *Triticum aestivum* through a meristem infection with 33% transformation efficiency. Yasmeen *et al.* (2009) reported 23% transformation frequency by using the *in planta* system for a tomato, and Tjokrokusumo *et al.* (2000) reached a 10% transformation rate in a *Petunia hybrid* by stigma infection.

Regarding the high impact of the *Agrobacterium* infected tissue type on the success of this technique, we used two different tissues, the shoot apical meristem and ovary. Various plant tissues and organs such as microspores, seeds, internodes, and shoot apical meristems have been utilised by researchers for the *in planta* transformation method (Arthikala *et al.* 2014). However, most researchers used SAM for the generation of stable transgenic plants in this system regarding the fact that undifferentiated cells can normally change to differentiated cells and regenerate into plants. For example, an *in planta* system using meristematic tissues was utilised in cotton as a recalcitrant plant which shows a genotype-dependent transformation pattern and the stable transgenic seedlings were generated by 6.98% transformation efficiency (Kalbande & Patil 2016). However, according to the result in our study, the PCR analysis showed the integration of the transgene in 3 out of 150 (2%) primary transformants by the SAM inoculation. Considering the possibility of the chimeric plant production or the integration of T-DNA region containing the *GFP* gene to other cellular parts which surrounded the apical meristem of the germinated seeds, it can probably lead to the low percentage (2%) of the confirmed transgenic plants. In other words, many of the 150 surviving plants can be chimeras so they did not exhibit the *GFP* insertion. Besides, the transgene could be transferred into the already-differentiated cells around the apical meristem, so it led to the detection of the integrated gene and the subsequent *GFP* expression just in the lower part of the T0 plant. However, if the T-DNA is inserted into the meristematic cells, the transgene insertion and expression is detectable in the upper areal part of the seedling such as the young upper leaves that were tested. Production of the chimeras caused through the apical meristem transformation strategy was also observed and explained by Supartana *et al.* (2006). The low rate of positive transformation events by SAM might be due to the inaccuracy in the proper

meristematic region selection, the inappropriate growth stage of the undifferentiated meristem cells at the time of the infection, and most probably, the inefficiency of the tissue type we used for the transformation in this plant. So, we concluded that the SAM inoculation method is not an optimal method for the transformation of this plant.

Based on the facility of the flower ovary injection in the self-pollinated *Catharanthus* plant, we examined another method for generating the transgenic plants. As the results demonstrated, the rate of transgenic lines has been obviously increased by use of the ovary injection to about 12%. Based on Fisher's exact analysis test, a significant difference ($P < 0.05$) between the ovary and SAM procedures was observed in the confirmed transgenic lines which demonstrated the *GFP* integration with the ovary method superiority over the SAM one. The results revealed that six lines obtained from 50 seedlings showed the *GFP* gene integration. Besides, the probability of the chimeric plants could be rejected because the following generation or the T1 plant that originated from seeds of the injected ovary were examined. In addition to the improvement in the transformation efficiency, the *in vivo* transformation method in contrast to the *in vitro* techniques is quick, easy, inexpensive, and genotype-independent. In the present study, we reached putative transgenic lines in the ovary injection method in less than three months from the injection time that is a significantly short time compared to the standard tissue culture and regeneration process.

Apart from an increase in the transgene integration efficiency observed through the ovary injection procedure, much more healthy and developed plants were produced and survived by this method compared with the SAM inoculation. In addition, many of inoculated SAMs were missed through the soil transfer as they were very fragile and sensitive. In line with our result of 12% transformation efficiency, the *in planta* ovary injection has been previously used in soybean by Liu *et al.* (2009) and a maximum transformation frequency of 11% was obtained. Since the *in planta* method is a kind of an *in situ* reproductive transformation system, they used the pod-bearing rate trait to examine the transformation yield (Liu *et al.* 2009). Therefore, by using the ovary injection method, it is possible to reduce the laborious expensive confirmation tests while increasing the accuracy of the confirmation regarding the physiological trait examinations instead of the molecular tests. Considering this established ovary injection

system as a primary method for producing transgenic *Catharanthus* plants which is independent from tissue culture, researchers can develop this protocol by optimising different factors so that a higher rate of transformation (than 12%) can be achieved and applied in *Catharanthus* genetic engineering programmes. Besides, according to the result of this study, the *in planta* method through the ovary injection is introduced as a smooth, efficient, and reproducible method for generating stable transgenic lines in *C. roseus*.

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